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Department of Genetics

PROGRESS REPORT

(Research Grant No. E-2872)

IMMUNOGENETICS OF SALMONELLA

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Granted Period

September 1, 1959 thru August 31, 1962

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## SUMMARY STATEMENT

The main progress attained during the granted period is in the studies of the functional relationship of the genes which control the synthesis of flagellar antigen in Salmonella. Those genes are  $H_1$  and  $H_2$ , the determinant of phase-1 and phase-2 antigen type respectively;  $Vh_2$ , a regulator of the stability of  $H_2$ , consequently of antigenic phases;  $Ah_1$ , a controller of  $H_1$  activity; and Fla genes, regulators of the quantity of the flagellar antigen produced.

$H_1$  and  $H_2$  were found not only to determine the antigenicity of flagella but also flagellar shape and sensitivity to certain motility phage. They are presumed to be the primary structural determinants of flagellar protein in phase-1 and phase-2 respectively. Antigen type mutants and H inactive mutants have been accumulated, and genetic fine structure analysis of H-genes is in progress. In parallel, chemical comparative analysis of flagellar antigen substances of those mutants is on the way. A method to purify flagellar antigen substance by cellulose chromatography was invented.

The general rule of functional interactions between  $H_1$  and  $H_2$  (: epistasis of active  $H_2$  over  $H_1$  and the reversible change of  $H_2$  activity in phase variation) in diphasic strains was extended to phase-2 monophasic strains and an unequal recombinant on H-genes. The epistasis and the reversible change were found to be not antigen type specific but locus specific. The function of  $H_1$  and  $H_2$  are duplicate in case of the antigenic specificity is disregarded. It was further indicated that the presence of  $Vh_2$  on the vicinity of  $H_2$  is essential for the unstabilization of the  $H_2$ .  $Ah_1$  is a complementation unit which is closely linked to  $H_1$ . Its function is phase specific. Fla<sup>-</sup> mutants, which have lost the ability to produce flagella in both antigenic phases, were classified into ten cistrons by complementation test. A type of Fla<sup>-</sup> mutants was detected which produced the cross reacting material of flagellar antigen in their cells.

FULL STATEMENT OF PROGRESS

1. PUBLICATION AND ORAL REPORTS

a). Published

Iino, T., 1960, Subunits of  $H_1$  gene in Salmonella. Ann. Rep. Natl. Inst. Genetics (Japan) 10:111-112.

———, 1960, Unequal recombination in Salmonella. ibid. 10:112-113.

b). Accepted for publication

Iino, T., 1961, Genetics of O-H variation in Salmonella. Japanese Journal of Genetics

———, 1961, A stabilizer of antigenic phases in Salmonella. Genetics

———, 1961, Abnormal homology of flagellar phases in Salmonella. ibid.

———, 1961, Curly flagellar mutants in Salmonella. Journal of General Microbiology

c). Oral reports

Haruna, I. and T. Iino, Separation and purification of antigenic substance of Salmonella flagella. 34th Meeting of the Japanese Biochemical Society (Nov. 4, 1961, at Osaka)

Iino, T., Unequal recombination in Salmonella. 31st Meeting of the Genetics Society of Japan (Nov. 5, 1959, at Osaka)

———, Genetic aspects on the phylogeny of Salmonella serotype. 33rd Meeting of the Bacteriological Society of Japan (July 20, 1960, at Sapporo)

———, Genetics of Salmonella in reference to fine structure analysis. 32nd Meeting of the Genetics Society of Japan (Nov. 1, 1960, at Fukuoka)

Iino, T., Curly flagellar mutants in Salmonella. 33rd Meeting of the Genetics Society of Japan (Sept 3, 1961, at Sendai)

Sasaki, I., Multiplication of chi-phage in H-type Salmonella. 8th Meeting of the Japanese Virologist (Oct.-29, 1960, at Kyoto)

———, Host range mutation of chi-phage. 33rd Meeting of the Genetics Society of Japan (Sept. 3, 1961, at Sendai)

———, S. Tsuji and T. Iino, Relationship between chi-phage and H-antigen of Salmonella. 13th Meeting Kanto Branch, Bacteriological Society of Japan (Apr. 27, 1960, at Yokohama)

## 2. STAFFING

Tetsuo Iino, Ph.D., Researcher (principal investigator), Sept. 1959-, Spent 75% of the period on this project.

Shigekatsu Tsuji, B.Sc., Research fellow, Oct. 1959 thru Apr. '60, Half time.

Itiro Sasaki, M.D., Researcher (associate), Dec. 1959-, Full time.

Hideo Hirokawa, M.Sc., Researcher (assistant), Half time July 1960 thru Apr. '61, Full time May 1961-.

Masatoshi Enomoto, M.Sc., Researcher (assistant), Apr. 1961-, Half time.

Ichiro Haruma, D.Sc., Researcher of the Institute of Virus Research, Kyoto University, Kyoto, Japan, cooperated with us the chemical parts of the investigation since Jan. 1960.

### 3. PROGRESS IN RESEARCH

The project aims to clarify the genetic mechanism of specific antigen (especially H-antigen) production and the antigenic variations in Salmonella. The investigation has been focused in the following three subjects: I). genetics of antigenic phase variation (Iino, Sasaki, Tsuji); II). genetic analysis on the biosynthesis of flagellar antigen (Iino, Hirokawa, Enomoto); III). genetic fine structure analysis of H-antigen determinants (Iino, Haruna).

#### I). Genetics of antigenic phase variation

The preceding studies had shown that H<sub>1</sub>- and H<sub>2</sub>-gene are the determinants of H-antigen type in phase-1 and phase-2 respectively in Salmonella, and the antigenic phase variation in a diphasic strain occurs by the oscillatory change of H<sub>2</sub> between active and inactive states. In other words, H<sub>2</sub> is epistatic over H<sub>1</sub> and changes its inherent activity reversibly: when both H<sub>1</sub> and H<sub>2</sub> are in active state in a cell, the cell expresses the phase-2 antigen; when H<sub>2</sub> is inactivated, the H<sub>1</sub> determined phase-1 antigen is produced.

During the granted period, several new informations have been obtained as regards antigenic phase variation.

#### (1). Interaction between H-genes

Phase-2 monophasic mutants of diphasic S. typhimurium were found to perform O-H variation in place of phase variation: phase-1 is O-type and phase-2 is H-type. Transductional analysis demonstrated that both H<sub>1</sub> and H<sub>2</sub> are inactive in phase-1 and only H<sub>2</sub> is active in phase-2 in such mutants. These results indicate that the function of H<sub>1</sub> and H<sub>2</sub> is duplicate in flagellar synthesis in cases that the antigenic specificity is disregarded. The inactivation of H<sub>1</sub> is caused by mutation in a cistron, called Ah<sub>1</sub>, which is closely linked to H<sub>1</sub> but separable from it by recombination. H<sub>1</sub> and Ah<sub>1</sub> show at least partial complementation in cis-trans test.

(2). A stabilizer of phase variation

Genetic analysis of a stability controller,  $Vh_2$ , of phase variation was continued. In S. abortus-equi, an allele  $Vh_2^-$  stabilizes  $H_2$  in its existing state, whether inactive or active, and produces phase-1 or phase-2 monophasic types respectively.  $Vh_2$  is transduced linked to  $H_2$ . The stability of the antigenic phases of the strains which carry  $Vh_2^-$  is exceedingly high, and such strains may be used as excellent materials for the production of specific anti-H serums.

(3). Anomalous homology of flagellar phases

An abnormal H-antigen type recombinant which alternatively expresses phase-1 antigens of both donor (i) and recipient (b) was obtained from a transduction between S. typhimurium and S. abony. In the transductional progeny test, the duplicated phase-1 antigen type determinant  $H_1^b$  of the recombinant behaves as an allelic locus of phase-2 antigen type determinant  $H_2$ . The recombinant is presumed to be originated by unequal recombination:  $H_2$  locus is replaced by  $H_1$  of the donor in the transduction. This phenomenon suggests the phylogenical homology between  $H_1$  and  $H_2$ : one of them might have originated by duplication and translocation of the other: the structural differentiation might have occurred between them thereafter. It also indicates that the effect of  $Vh_2$  is not antigen type specific but locus specific.

(4). The effect of various chemicals on phase variation

The chemical agents which can alter the frequency of phase variation have been looked for. The chemicals include bromouracil, fluorouracil, chloromycetin, penicillin, streptomycin, acriflavin, formaldehyde, Na-azide and methyl green. The significant effect of those chemicals on phase variation has not been detected. The survey is being continued and the continuous culture apparatus is being under construction for this purpose.

(5). Changes associated with phase variation

Other than the specificity of H-antigen, the following two characters were found to be determined by  $H_1$  and  $H_2$ , and the changes of those characters associate with antigenic phase variation.

a). curly flagellar shape

Flagella having an wavelength of half that found in the wild type have been called curly flagella. A curly flagellar mutant obtained from a strain of S. typhimurium is unstable and repeatedly dissociates curly and normal subclones. Examination of the flagellar antigens of the normal and curly flagellar subclones demonstrated that the change in flagellar shape correspond exactly with phase variation: subclones with curly flagella are always in phase-1 (i-antigen) and those with normal flagella are in phase-2 (1.2-antigen).

In transduction from a normal flagellar strain to the curly phase-1 strain, transductional clones with normal flagella were isolated. The transductional clones showed the antigen of the donor in phase-1 and that of the recipient in phase-2. From this result it is concluded that the phase-1 curly determinant is closely associated with the phase-1 antigen type determinant,  $H_1$ .

Seven curly mutants were obtained from a strain of S. abortus-equi. Transductional analysis with these strains showed that the phase-2 curly determinant is closely associated with the phase-2 antigen type determinant  $H_2$ ; and the phase-1 curly determinant with  $H_1$ .

In cross absorption experiments with antiserum prepared against flagella of either normal or its curly mutant, no antigenic difference between normal and curly flagella could be detected.

Attempts to obtain recombination by transduction between the curly flagellar determinants in each phases have been unsuccessful; this suggests that the mutant sites of the curly types are very closely linked or identical in each phases.

b). sensitivity to a motility phage, chi

Chi-phage has been known to attack motile *Salmonella* except those which have g-antigenic flagella. Mutants of *S. typhimurium* were found which are resistant against chi-phage in motile phase-2 (1.2-antigen), while they retain the sensitivity in phase-1 (i-antigen). A host range mutant of chi-phage can attack both the flagella-less and paralyzed mutants obtained from the original strain of the resistant mutants. The resistant phenotype in phase-2 of the mutant strains seems to be expressed by interaction of  $H_2$  gene and some other genetic factor(s): when the  $H_2^{1.2}$  of a resistant mutant is replaced with another  $H_2$  allele, for example  $H_2^{enr}$  of *S. abortus-equi* by transduction, the transductional clones become sensitive in phase-2 as well as in phase-1; while 1.2-antigen type of transductional clones obtained by transduction of the reverse direction remained sensitive. The more detailed genetic analysis is in progress.

The association of these mutant characters with H-genes may indicate that  $H_1$  and  $H_2$  are the primary structural determinants of flagellar protein in phase-1 and phase-2 respectively; a mutant in  $H_1$  or  $H_2$  may cause an altered configuration of flagellar protein, resulting in a change of antigenic type, flagellar shape or a sensitivity to the motility phage, or it may cause the failure of flagellar morphogenesis.

II). Genetic analysis on the biosynthesis of flagellar antigen

(1). Complementation between  $Fla^-$  genes

$Fla$  genes are regulators of flagellar synthesis. They do not determine or modify the specificity of flagellar antigen but regulate the amount of flagella produced.

Complementation analysis was carried out extensively on  $Fla^-$  mutants obtained from *S. typhimurium* and *S. abortus-equi*. The production of trails in transduction between  $Fla^-$  mutants was used as the criterium of complementation. 53 of them were classified into ten groups (cistrons). In two of these groups, partial



complementation was observed. The parallelism of the complementation map and the linkage map based on the recombination frequency is under investigation.

(2). Production of the substance cross reacting with flagellar antigen in Fla<sup>-</sup> mutants

The antigenicity of twenty one Fla<sup>-</sup> mutants, which are highly stable and represent all the complementation units, were examined. They all gave negative result in agglutination test with anti-H serum; while, when they were injected to rabbits, two of them caused to produce anti-H sera. The antigenic substance was isolated from the sonicated cells of these two mutants by means of cellulose chromatography (ref. III). The chemical comparative analysis of the H-antigen substance of the Fla<sup>+</sup> wild strain and the cross reacting material found in these Fla<sup>-</sup> mutants is in progress.

(3). Test of syntrophism between Fla<sup>-</sup> mutants

The possibility of syntrophic recovery of flagellar production was examined with mixed cultures of two complementary Fla<sup>-</sup> mutants. None of the combinations tested recovered the ability of flagellar production.

(4). Temperature mutants on flagellar production

TM2 strain of S. typhimurium can grow at 42C, but cannot produce flagella at the temperature higher than 40C. Two temperature mutants which can produce flagella at 40C thru 45C as well as below 40C were isolated from TM2. The temperature resistant character was not transduced linked to any known Fla genes. Detailed analysis on the nature of the temperature resistance is under investigation.

III). Genetic fine structure analysis of H-antigen determinants

(1). Genetic analysis

H<sub>2</sub> inactive mutants and the partial antigen type mutants of H<sub>2</sub><sup>1.2</sup> and H<sub>2</sub><sup>enx</sup> alleles have been accumulated from a strain of S. abortus-equi SL23 (enx-antigen) and its transductional derivative SJ25 (1.2-antigen). Semisolid nutrient-gelatine-agar medium supple-

mented with anti-enx (or -1.2) serum was used for the selection: an H<sub>2</sub> inactive mutant clone produces a swarm with flagellar antigen-a on the selective medium. The recombination analysis with these mutants is on the way of accumulation of genetic data. Curly flagellar mutants and the mutants resistant to chi-phage (reported in I.5) were also adopted for the analysis.

## (2). Isolation and purification of flagellar antigen substance

In parallel with the genetic fine structure analysis of H<sub>2</sub> gene, chemical studies of flagellar antigen substance was started.

The method to purify flagellar antigen substance by cellulose chromatography was invented. The outline of the procedure is as follows: sonicate the saline suspension of the bacterial cells for 1 minute at 20 kc; the suspension was centrifuged and the cell free supernatant was acidified (pH 3.5) by 1/10 N HCl and centrifuged; the supernatant was dialysed and adsorbed to DEAE cellulose column with 0.005 M borate buffer; the adsorbed materials were fractionated by gradient elution with changing pH and salt concentration; antigenicity of each fraction was examined by gel-diffusion test; flagellar antigen substance was isolated to a fraction. The flagellar antigen substances of the enx-type flagella, 1.2-type flagella and curly mutant flagella came to the same fraction of the chromatogram.

The advantage of this method to the previous one, in which flagella were purified first by differential centrifugation, is that we can detect flagellar substance in bacterial cells before it is organized to the visible filamentous organelle. By the application of this method flagellar antigen substance produced by some flagella-less mutants was isolated (reported in II.2)